Name _____

Investigation 8: Biotechnology
Bacterial Transformation

Objective

In this laboratory investigation, plasmids containing fragments of foreign DNA will be used to transform *Escherichia coli* cells, conferring antibiotic (ampicillin) resistance, as well as an inducible gene GFP (Green Fluorescent Protein) from the bioluminescent jellyfish, *Aequorea Victoria*.

Background

Are genetically modified foods safe? There is ongoing debate about whether it is safe to eat fruit and vegetables that are genetically modified to contain toxins that ward off pests. For instance, biotechnologists have succeeded in inserting a gene (Bt) from the bacterium *Bacillus thuringiensis* into the corn genome. When expressed, the Bt toxin kills caterpillars and controls earworms that damage corn, but is the corn safe for human consumption?

Genetic information passed from parent to offspring via DNA provides for continuity of life. In order for information in DNA to direct cellular activities, it must be transcribed into RNA. Some of the RNAs are used immediately for ribosomes or to control other cellular processes. Other RNAs are translated into proteins that have important roles in determining metabolism and development, i.e., cellular activities and phenotypes (traits). When the DNA of a cell changes, the RNAs and proteins they produce often change, which in turn changes how that cell functions.

DNA inside a cell can change several ways. It can be mutated, either spontaneously or after the DNA replication machinery makes an error. Biotechnologists may cause an intentional mutation in a cell's own DNA as a way to change how that cell behaves. The most powerful tool biotechnologists have, though, is the ability to transfer DNA from one organism to another and make it function there. With this tool, they can make cells produce novel protein products the cells did not make previously.

Examples of this powerful tool are all around us. Insulin that people take to control their blood sugar levels is often made from engineered bacteria. Some vaccines, as well as enzymes used for manufacturing denim jeans, are also made using engineered cells. In the near future, engineered bacteria and other cells being developed could help clean up spilled oil or chemicals, produce fuel for cars and trucks, and even store excess carbon dioxide to help slow global climate change. Can you think of other possible applications of genetic engineering? However, biotechnology and human manipulation of DNA raise several ethical, social, and medical issues, such as the safety of genetically modified foods.

This biotechnology depends on plasmids, small circles of DNA that were found first in bacteria. Plasmids allow molecular biologists to manipulate genetic information in a laboratory setting to understand more fully how DNA operates. Plasmids also let us move DNA from one bacterium to another easily.

In this investigation, you will learn how to transform *Escherichia coli* (*E. coli*) bacteria with DNA it has not possessed before so that it expresses new genetic information. Bacterial cells that are able to take up exogenous (external) genetic material are said to be "competent" and are capable of being transformed. You can also calculate transformation efficiency to find out how well the *E. coli* took up the "foreign" DNA.

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Bacteria, Transformation, and Plasmids

DNA provides the instructions necessary for the survival, growth, and reproduction of an organism. When genetic information changes, either through natural processes or genetic engineering, the results may be observable in the organism. These changes may be advantageous for the long-term survival and evolution of a species, but it also may be disadvantageous to the individuals who possess the different genetic information.

In bacteria, genetic variation does not happen by mutation alone. It also can be introduced through the lateral (horizontal) transfer of genetic material between cells. In nature genes can be transferred between bacteria in three ways; conjugation, transduction, or transformation. **Conjugation** is a mating process during which genetic material is directly transferred from one bacterium to another of a different mating type. **Transduction** requires the presence of a vector (such as a virus) to transfer small pieces of DNA from one bacterium to another. Bacterial **transformation** involves transfer of genetic information into a cell by direct uptake of the "naked" DNA from the environment outside the cell. During gene transfer, the uptake and expression of foreign DNA by a recipient bacterium can result in conferring a particular trait to a recipient lacking that trait. (We studied transformation in a different context when discussing an experiment conducted by Frederick Griffith. Recall that he mixed heat-killed remains of a pathogenic strain of bacteria with living cells of a harmless strain, resulting in some of the living cells becoming pathogenic. Little did Griffith know that his work would provide a foundation for genetic engineering and recombinant DNA technology in the 21st century!)

Genetic transformation of bacteria most often occurs when bacteria take up plasmids from their environment. Plasmids are not part of the main DNA of a bacterium. They are small, circular pieces of DNA that usually contain genes for one or more traits that may be beneficial to survival. Certain plasmids, called R plasmids, carry genes for resistance to such antibiotics as ampicillin, kanamycin, or tetracycline. [Antibiotic-resistant bacteria are responsible for a number of human health concerns, such as methicillin-resistant Staphylococcus aureas (MRSA) infections.] Other plasmids code for an enzyme, toxin, or other protein that gives bacteria with that plasmid some survival advantage. In nature, bacteria may swap these beneficial plasmids from time to time. This process increases the variation between bacteria — variation that natural selection can act on. In the laboratory, scientists use plasmids to insert "genes of interest" into an organism to change the organism's phenotype, thus "transforming" the recipient cell. Using restriction enzymes, genes can be cut out of human, animal, or plant DNA and, using plasmids as vectors (carriers of genetic information), inserted into bacteria. If transformation is successful, the recipient bacteria will express the newly acquired genetic information in its phenotype. For instance, if these plasmid vectors also carry genes for antibiotic resistance or fluorescent proteins, transformed cells containing these plasmids can be easily selected from other cells without the foreign DNA (see Figures 1 and 2 on the next page).

Name _____

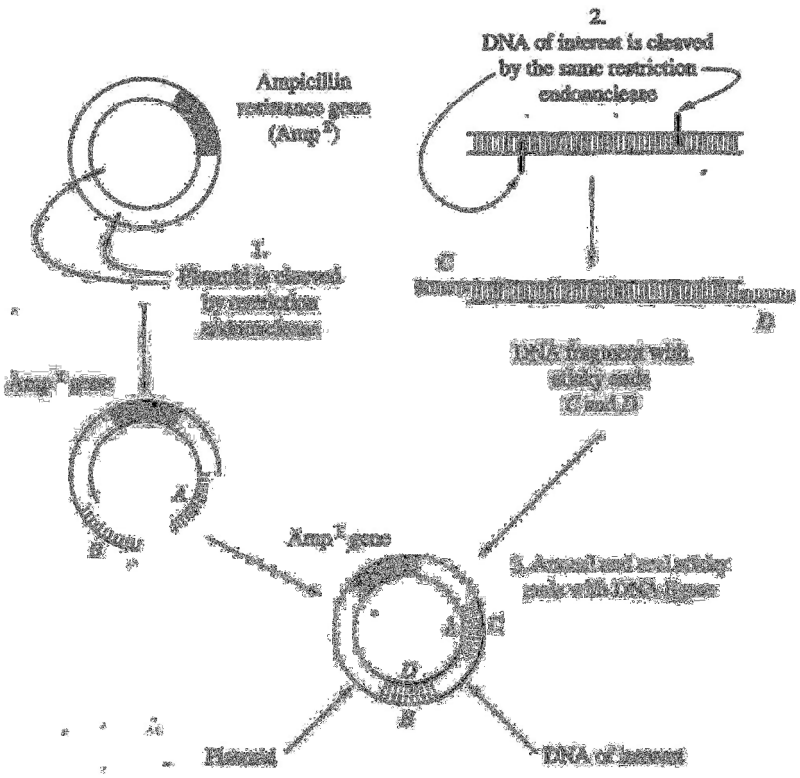
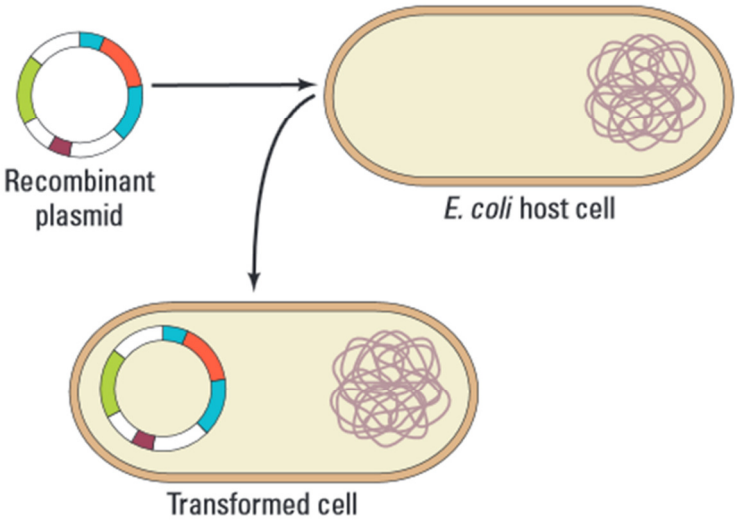


Figure 1 (left): Recombinant Plasmid Using a Restriction Endonuclease

Figure 2 (below): Transformation of Bacteria



In nature, the efficiency of transformation is low and limited to relatively few bacterial strains. Also, bacteria can take up DNA only at the end of logarithmic growth. At this time, the cells are said to be "competent." Competence can be induced in *E. coli* with carefully controlled chemical growth conditions. Once competent, the cells are ready to accept DNA that is introduced from another source. The number of bacteria that take up a plasmid successfully is called the "transformation efficiency."

To genetically transform an entire organism, you must insert the new gene into every cell in the organism. Thus, unicellular organisms, like bacteria, are better suited for total genetic transformation than are multicellular organisms. The bacterium *Escherichia coli* (*E. coli*) is an ideal organism for the molecular geneticist to manipulate and has been used extensively in recombinant DNA research. It is a common inhabitant of the human colon and can easily be grown in suspension culture in a nutrient medium such as Luria-Bertani (LB) agar/broth. *E. coli* reproduce very rapidly; a single microscopic cell can divide to form a visible colony with millions of cells overnight. Like all bacteria, *E. coli* has no nuclear envelope surrounding the bacterial chromosome and thus no true nucleus. All of the genes required for basic survival and reproduction are found in the single chromosome. Some *E. coli* cells also contain plasmids, small DNA molecules that carry genes for certain specialized functions, including resistance to specific drugs.

Name _____

Transformation Scheme

Most transformation protocols can be conceptualized as four major steps:

1. Preincubation: Cells are suspended in a solution of cations and incubated at 0°C. The cations are thought to complex with exposed phosphate heads of the phospholipids of the *E. coli* cell membrane. The low temperature freezes the cell membrane, stabilizing the distribution of the negatively charged phosphate heads.

2. Incubation: DNA is added, and the cell suspension is further incubated at 0°C. The cations are thought to neutralize negatively charged phosphates in the DNA and cell membrane. With these charged neutralized, the DNA molecule is free to pass through the cell membrane. (During this time, DNA becomes attached to the outer surface of the cells.)

3. Heat Shock: The cell/DNA suspension is briefly incubated at 42°C and then quickly returned to 0°C. The rapid temperature change creates a thermal imbalance (gradient) on either side of the *E. coli* membrane, which is thought to create a draft that sweeps plasmids into the cell. (This "heat-shock" step opens pores in the cell's membranes, allowing the DNA to enter some cells. The heat shock requires instantaneous transitions between cold to hot to cold.)

4. Recovery: LB broth is added to the DNA/cell suspension and incubated at 37°C (ideally with shaking) prior to plating on selective media. Transformed cells recover from the treatment, amplify the transformed plasmid, and begin to express the antibiotic resistance protein. (This recovery time with agitation gives the new genes introduced into the cells time to be transcribed and translated into proteins.)

Antibiotic Selection/Resistance

Ampicillin is the most practical antibiotic resistance marker for demonstration purposes, especially in the rapid transformation protocol described here. Ampicillin inhibits the growth of bacteria by interfering with the construction of the peptidoglycan layer and kills dividing cells that are assembling new cell walls. The *bla* gene, which is carried on the recombinant pGLO plasmid we are using, encodes an enzyme called β-lactamase, which disables the ampicillin molecule, conferring the ampicillin resistance phenotype (amp^r). β-lactamase not only disables ampicillin within the bacterial cell, but because it leaks through the cellular envelope, it also disables ampicillin in the surrounding medium. Thus, we can tell which cells took up the plasmid because only transformed bacteria, which now contain the *bla* gene and are therefore resistant to ampicillin, will grow on a plate that contains ampicillin.

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The pGLO system

To possess a trait, organisms need to both possess the necessary genes AND express them. The expression of a group of functionally related genes can be coordinately controlled by a single "on-off switch." This regulation takes place at a very specific location on the DNA template, called a promoter, where RNA polymerase sits down on the DNA and begins transcription of the gene. In bacteria, groups of related genes are often clustered together and transcribed into RNA from one promoter. These clusters of genes controlled by a single promoter are called operons.

Gene expression can be altered in response to changes in the environment. For example, *E. coli* bacteria produce three enzymes needed to digest the sugar arabinose as a food source. The genes that code for these enzymes can be switched on simply by adding the sugar arabinose to the cell's nutrient medium. However, so as not to waste energy making unnecessary enzymes, the genes which code for those enzymes can be turned off (and will therefore not be expressed) in the absence of arabinose.

The DNA code of the recombinant pGLO plasmid has been engineered to incorporate aspects of the arabinose operon (in addition to the gene for resistance to the antibiotic ampicillin). Both the promoter (P_{ara}) and the araC gene are present. However, the genes which code for arabinose catabolism have been replaced by the single gene which codes for Green Fluorescent Protein (GFP). Figure 3 shows the plasmid's relative gene locations. (ori indicates the origin of replication)

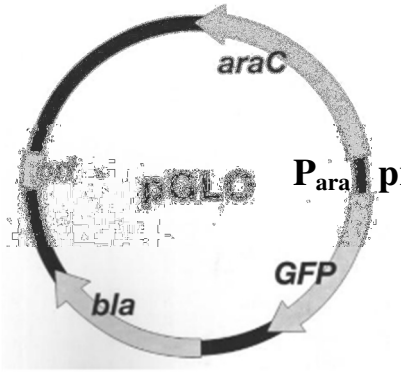

Figure 3: pGLO plasmid

The araC gene encodes a repressor protein that blocks transcription at the P_{ara} promoter in the absence of arabinose. But in the presence of arabinose, the sugar removes the repressor, promoting the binding of RNA polymerase which allows the transcription of the gfp gene, so that the Green Fluorescent Protein (GFP) is produced (Figure 4). Cells fluoresce brilliant green as they produce more and more GFP. In the absence of arabinose, araC no longer facilitates the binding of RNA polymerase and the GFP gene is not transcribed. When GFP is not made, bacteria colonies will appear to have the wild-type (natural) phenotype—of white colonies with no fluorescence.

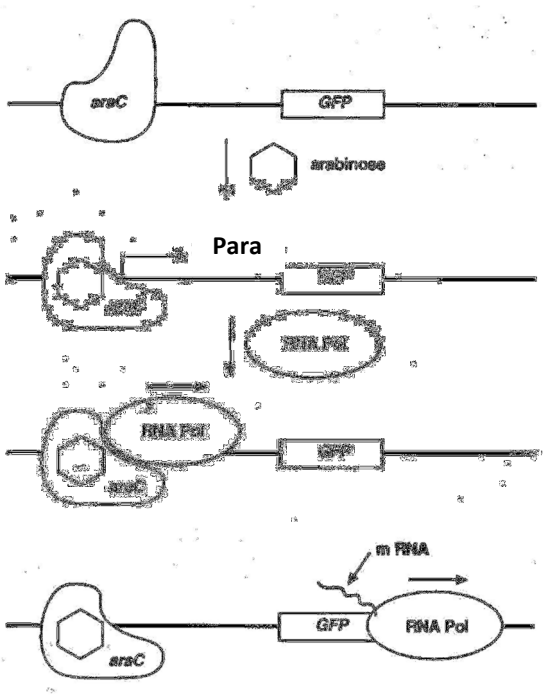

Figure 4: Expression of GFP

Selection for cells that have been transformed with pGLO DNA is accomplished by growth on antibiotic plates. Transformed cells will appear white (wild-type phenotype) on plates not containing arabinose, and fluorescent green when arabinose is included in the nutrient agar.

Name _____

Safety

BASIC STERILE TECHNIQUE

With any type of microbiology technique (i.e., working with and culturing bacteria), it is important not to introduce contaminating bacteria into the experiment. Because contaminating bacteria are ubiquitous and are found on fingertips, bench tops, lab tables, etc., it is important to avoid these contaminating surfaces. When working with the inoculation loops, bulb pipets, micropipettes, and agar plates, do not touch the round circle at the end of the loop, the tips of the pipets, or the surface of the agar plate, and do not place them onto contaminating surfaces. Be sure to wash your hands before beginning the procedure and after (and cover your sneezes!) Do NOT eat, drink, apply cosmetics, or use personal device in the lab.

WORKING WITH E. COLI

The host *E. coli* used in this investigation, the vector containing the recombinant GFP protein, and the subsequent transformants created by their combination are not pathogenic organisms like the *E. coli* O157:H7 strain that has been in the news. However, handling of this strain requires the use of appropriate microbiological and safety procedures. These practices include but are not limited to the following:

- Decontaminating work surfaces once a day and after any spill of viable material with a 10% household bleach solution
- Decontaminating all contaminated liquid or solid wastes before disposal [This can be done in an autoclave (20 minutes at 121°C) or in a 10% bleach solution (soaked for 20 minutes).]
- Washing your hands after handling organisms containing recombinant DNA and before leaving the lab
- Wearing protective eyewear and disposable gloves
- Not eating, drinking, applying cosmetics, or using personal electronic devices, such as iPods and cell phones, in the lab

Micropipetting Review

First Step: Measuring

- **Choose a micropipette whose range spans the desired volume.**
- Set the micropipette to the desired volume by turning the plunger knob. **Don't force past the pipette's limits, as this breaks the pipette!**
- Place a tip on the micropipette, matching tip and plunger colors.
- Depress plunger to the **FIRST STOP** and **HOLD**.
- Place the tip into the liquid.
- **Slowly** release the plunger, **keeping tip in liquid**.

Second Step: Dispensing

- Place tip against side of tube, near bottom.
- Depress plunger to the **SECOND STOP** and **HOLD**.
- Remove tip from tube **while holding down plunger**.
- Release plunger.
- Throw away used tip using the ejector button. **Use a new tip each time you pipet.**

Remember,
1st Step, 1st Stop. 2nd Step, 2nd Stop.

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Pre-Lab Questions

1. Describe at least 2 practical applications of genetic engineering.
   -
   -

2. Identify AND explain the three ways genes can be transferred between bacteria
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   -
   -

3. What are plasmids AND what is their function in bacteria?

4. What does it mean if a cell is "competent"? (ie-What are "competent" cells capable of doing?)

Name _____

5. Explain three reasons prokaryotic organisms are better than eukaryotic organisms for investigating genetic information.
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   -
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6. What is the purpose of "heat-shocking" the cells by instantly transitioning them from cold to hot to cold?

7. How does the ampicillin plate allow you to determine which cells have been transformed?

8. Even if the cell has been transformed and now possesses the gfp gene, in order to produce the green fluorescent protein (GFP) the gene still needs to be expressed. What needs to be added to the cell's nutrient medium for the gene for GFP to be turned on?

9. What advantage would there be for an organism to be able to turn on or off particular genes in response to certain conditions?

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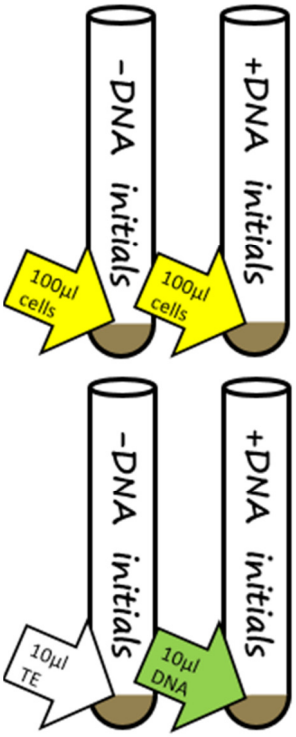
Procedure

Session 1: Transforming the Bacteria
Mixing bacterial cells and DNA under transformation conditions to introduce the engineered pGLO plasmid into *E. coli* cells

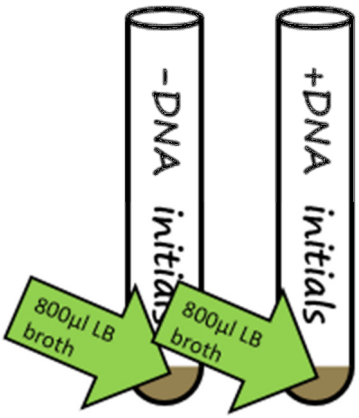
- Label your Transformation Culture Tubes
  1. Use a lab marker to label two 15mL round-bottom culture tubes:
     - -DNA & your initials
     - +DNA & your initials
  2. Place these tubes in your ice cup to chill
     - It is VERY important that the transformation reactions be kept cold. Don't handle these tubes or have them out of the ice for more than a few seconds at a time.



- Making the Transformation Mixtures
  3. To **each** tube, add **100 µl** of competent *E. coli* **cells**
     - Provided in a microtube in your ice cup
     - Pipet slowly (the cells are fragile)
     - Carefully deposit the drop of cells to the very bottom of the tube
     - Keep tubes on ice
     - Promptly replace the snap-on caps to avoid contamination by bacteria and fungi in the air
     - Don't forget: always use a fresh pipet tip each time
  4. To the **–DNA** tube, add **10 µl** of **TE Buffer** directly to the drop of cells
  5. To the **+DNA** tube, add **10 µl** of 5ng/µl pGLO plasmid **DNA** directly to the drop of cells
- Cold-Incubating the Transformation Mixtures
  6. Gently tap the bottom of each tube to gently mix the cells and solutions
  7. Incubate on ice for 15 minutes
     - During this time, DNA becomes attached to the outer surface of the cells
- Heat-Shocking the Transformation Mixtures
  8. Bring your ice cup with the two culture tubes to the 42ºC heat block
  9. Quickly place your pair of tubes into the heat block. Note the time
  10. After exactly 45 seconds, quickly remove your pair of tubes and immediately place them back in your ice cup for at least one minute
      - This "heat-shock" step opens pores in the cell's membranes, allowing the DNA to enter some cells. The heat shock requires instantaneous transitions between cold to hot to cold.

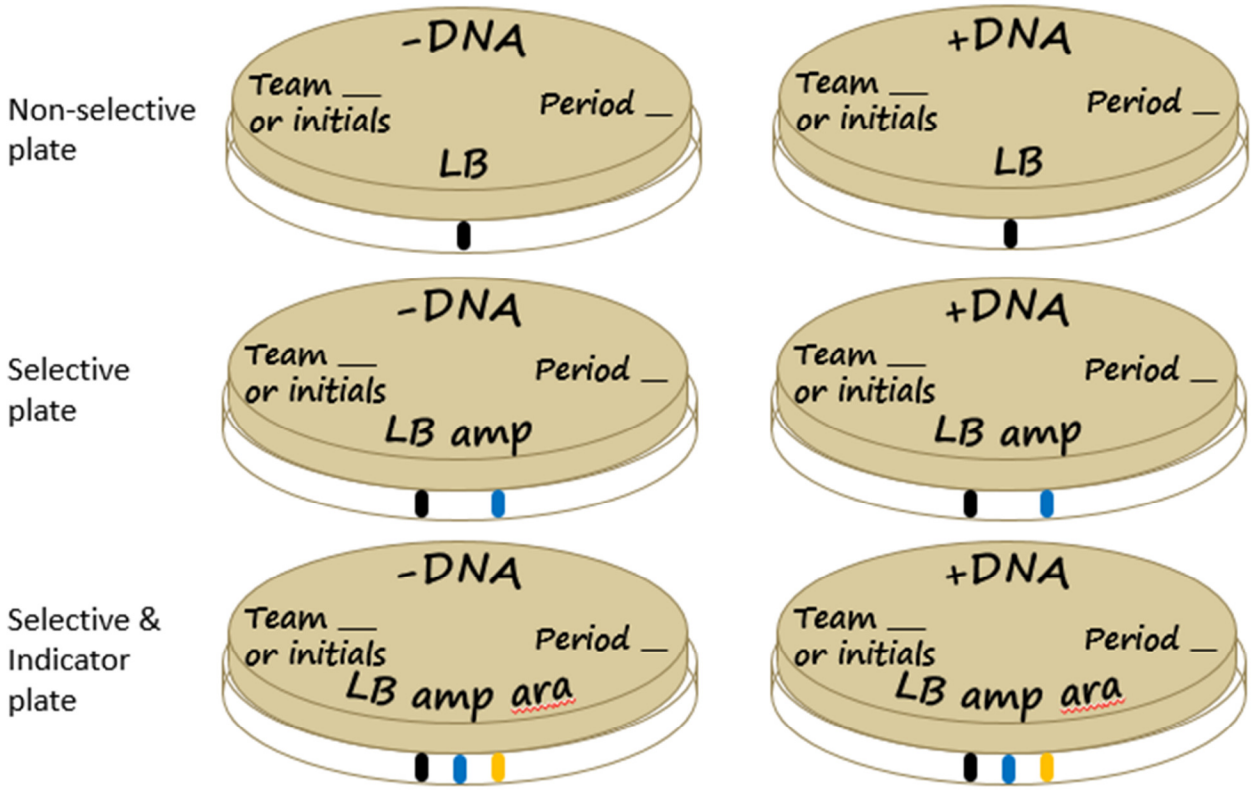
Name _____

- Initial Cell Culture: Recover and *bla* Gene Expression
  11. Add 800 µl of LB broth to each culture tube
      - Don't forget: always use a fresh pipet tip each time!
      - Replace the caps promptly to avoid contamination
  12. Tap the bottom of the tube to mix
  13. Place the tubes into the foam adapter mounted on a vortex mixer
      - Your samples will be agitated at room temperature for about 45 minutes. This gives the new genes (on the plasmid DNA) introduced into the cells time to be transcribed and translated into proteins



Session 2: Spread-Plating the Transformation Cultures
Growing the transformation cultures on non-selective, selective, and indicator plates

- Labeling the Plates
  1. Keep your plates agar-side up and label them as shown in the diagram below.



Name _____

- Spread-Plating
    2. Your foil packet contains two sterile yellow spreaders. Feel the foil packet and find the end shaped like a triangle. Carefully open the foil at the stick (not the triangle!) end, keeping the triangle ends covered with foil. Keep the spreader in the opened pack for now.
    3. Turn your three **–DNA** plates over (agar side on bottom) and apply 200 µl of your **–DNA** culture to **each** of the three **–DNA** plates (remember to use a fresh pipet tip each time!)
        - LB
        - LB amp
        - LB amp ara
    4. Remove one spreader from the pack (keep the other spreader covered) and use it to **gently** spread the liquid across the entire surface of each plate, turning the plate as you spread. **Don't press too hard, or the agar will tear.** Place the used spreader in the collection bin.
    5. Repeat using the other spreader to apply the **+DNA** culture to each of the three **+DNA** plates.
- Incubating Your Cultures
    6. Allow your plates to sit, agar side down, for few minutes to allow the liquid to absorb into the agar.
    7. Tape your set of six plates together using colored lab tape.
    8. Label the tape with your initials.
    9. Place your set of six plates agar side up in the 37ºC incubator for an overnight incubation.
- Making Predictions
    10. Predict what you expect each plate to look like tomorrow by completing Table 1 in the results section

Session 3: Interpreting Results

Examining for evidence of transformation and recombinant gene expression

- Examining Your Plates
    1. Record the appearance of each plate by completing Table 2 in the results section
        - Turn out the room lights and hold the UV lamp over your plates (Do NOT look directly into the UV lamp)
        - Record which of your plates have colonies that glow green

- Analysis of Results
    2. Practice calculating transformation efficiency by solving the problems given
    3. Explain what can be concluded from your results by answering the post-lab questions

Name _____

Results

Table 1: Predictions

	- DNA			+DNA		
	Grow?	Lawn or Colonies?	Glow under UV light?	Grow?	Lawn or Colonies?	Glow under UV light?
LB						
LB amp						
LB amp ara						

Table 2: Observations

	- DNA			+DNA		
	Grow?	Lawn or Colonies?	Glow under UV light?	Grow?	Lawn or Colonies?	Glow under UV light?
LB						
LB amp						
LB amp ara						

Name _____

Calculating Transformation Efficiency

Calculating transformation efficiency gives you an indication of how effective you were in getting plasmids carrying new information into host bacterial cells. It can be calculated using the following formula:

$$\text{Transformation efficiency} = \frac{\text{Total number of colonies growing on the agar plate}}{\text{Amount of DNA spread on the agar plate (in µg)}}$$

The most direct way to determine the total number of bacteria that were transformed with the pGLO plasmid is to count the colonies on the LB/amp/ara plate. In addition, since not all of the plasmid DNA added to the bacterial cells got transferred to the agar plate, you need to determine how much DNA was actually spread onto the LB/amp/ara plate. To calculate this, you will multiply the total amount of plasmid DNA used in the transformation times the fraction of DNA you spread on the LB/amp/ara plate.

a. Calculate total amount (mass) of plasmid DNA

Concentration of plasmid DNA (in µg/µL) x Volume of plasmid DNA added (in µL) = µg plasmid DNA

b. Calculate the fraction of plasmid DNA that got spread onto the LB/amp plate

$$\frac{\text{Volume spread on the LB/amp plate (µL)}}{\text{Total sample volume in test tube (µL)}} = \text{Fraction of DNA used}$$

c. Calculate the micrograms of plasmid DNA you spread on the LB/amp plate

Total amount of DNA used in µg (from part a) x Fraction of DNA used (from part b) = DNA spread in µg

d. Calculate transformation efficiency by dividing the number of colonies on the +DNA LB/amp plate by the amount of DNA spread on the plate

$$\frac{\text{Number of colonies on the +DNA LB/amp plate}}{\text{DNA spread on plate in µg (from part c)}} = \text{Transformation efficiency (in \# colonies/ µg of plasmid)}$$

Name _____

Sample Problem:
Let's say we added 10 µL of 0.005 µg/µL of plasmid DNA to a tube of *E. coli*. The bacteria were suspended in 300 µL of cold calcium chloride. After heat shock, 300 µL of Luria broth was added to the tube. 150 µL of cell suspension was placed on agar plates that were treated with an antibiotic. The plates were then incubated at 37ºC for 24 hours. 35 colonies were counted on the plate. What is the transformation efficiency?

a. 0.005 µg/µL X 10 µL = 0.05 µg plasmid DNA

b. $\frac{\text{150 µL spread on plate}}{\text{(300 µL calcium chloride + 300 µL Luria broth + 10 µL plasmid DNA)}} = 0.25$

c. 0.05 µg plasmid DNA (from part a) X 0.25 (from part b) = 0.0125 µg plasmid DNA spread on plate

d. $\frac{\text{35 colonies}}{\text{0.0125 µg plasmid}} = 2800 \text{ colonies / µg of plasmid} = 2.8 \text{ X } 10^3 \text{ colonies/ µg plasmid}$

1. Let's say you added 10 µL of plasmid DNA at a concentration of 0.08 µg/µL of to a tube of *E. coli*. The bacteria were suspended in 250 µL of cold calcium chloride. After heat shock, 250 µL of Luria broth was added to the tube. You then spread 100 µL of cells containing pGLO DNA on agar plates that were treated with ampicillin and arabinose. After the plates were incubated for 24 hours, you counted 190 colonies on the plate. What is the transformation efficiency?

2. Imagine you have plasmid DNA at a concentration of 0.290 µg/µL. You add 4 µL of this plasmid DNA to 50 µL of competent cells. After the appropriate steps, 100 µL of Luria broth are added to the cells. The cells are incubated for one hour before a 50 µL sample is spread on a selective plate. Following overnight incubation, 346 colonies are present on the plate. What is the transformation efficiency?

Name _____

3. Calculate the transformation efficiency of the following experiment using the information and results listed below:

- DNA plasmid concentration: 0.08 µg/µL
- 10 µL of plasmid added to culture
- 250 µL $CaCl_2$ transformation solution
- 250 µL LB broth
- 100 µL cells spread on agar
- 227 colonies of transformants

4. Calculate the transformation efficiency of the following experiment using the information and results listed below:

- DNA plasmid concentration: 0.005 µg/µL
- 10 µL of plasmid added to culture
- 200 µL $CaCl_2$ transformation solution
- 250 µL LB broth
- 100 µL cells spread on agar
- 50 colonies of transformants

5. Calculate the transformation efficiency of the following experiment using the information and results listed below:

- DNA plasmid concentration: 0.004 µg/µL
- 10 µL of plasmid added to culture
- 200 µL $CaCl_2$ transformation solution
- 250 µL LB broth
- 100 µL cells spread on agar
- 50 colonies of transformants

Name _____

Post-Lab Questions

1. What is the purpose of having a set of plates without the addition of plasmid DNA?

2. What is the purpose of having ampicillin on the plates?

3. What two factors must be present in the bacteria's environment for you to see the green color? (Hint: one factor is in the plate and the other factor is in how you look at the bacteria)

4. Did your predictions match your results? Explain the logic used to determine on which plates the bacteria should
   - Grow at all
   - Show less growth and only appear as colonies
   - Glow

5. Sometimes our results do not match our predictions (even when our logic is correct) due to experimental errors. Identify at least 3 possible sources of error in the transformation procedure that could affect the results.
   -
   -
   -

Name _____

6. In a molecular biology laboratory, a student obtained competent *E. coli* cells and used a common transformation procedure to induce the uptake of plasmid DNA with a gene for resistance to the antibiotic kanamycin. The results below were obtained.

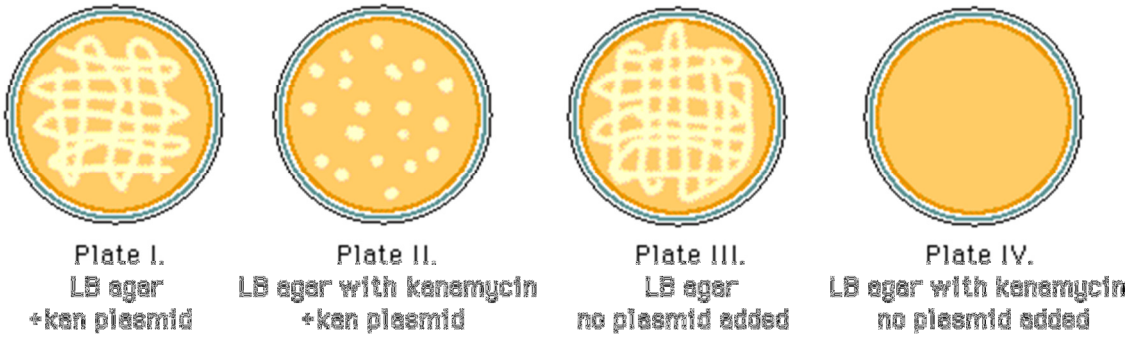


Plate I. LB agar +kan plasmid

Plate II. LB agar with kanamycin +kan plasmid

Plate III. LB agar no plasmid added

Plate IV. LB agar with kanamycin no plasmid added

- On which petri dish do only transformed cells grow?
- Which plate is used as a control to show that nontransformed *E. coli* will NOT grow in the presence of kanamycin?

7. A student has forgotten which antibiotic plasmid she used in her E. coli transformation. It could have been kanamycin, ampicillin, or tetracycline. She decides to make up a special set of plates to determine the type of antibiotic used. The plates below show the results of the test.

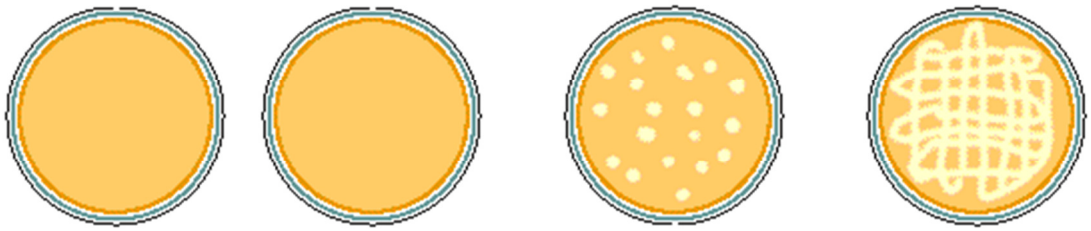


Plate I. LB agar with kanamycin

Plate II. LB agar with ampicillin

Plate III. LB agar with tetracycline

Plate IV. LB agar

- Which antibiotic plasmid has been used? Explain your reasoning